Universal assay for measuring vertebrate telomeres by real-time quantitative PCR

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Abstract

The role of telomere dynamics in health and aging has been facilitated by the development of qPCR-based telomere length measurements. However, the widespread application of this approach has been limited because of the challenge of developing appropriate reference primers in non-model organisms with genomes that have not been sequenced. Here we develop reference primers to highly conserved DNA elements and validate their use in qPCR-based telomere length measurements across the vertebrate tree of life.

Telomeres are the structures at the end of chromosomes that are comprised of proteins bound to repetitive DNA sequences. Telomeres protect the ends of linear chromosomes and provide several important cellular functions[1]. The length of telomere DNA shortens at each cell division, and telomere shortening eventually leads to cellular senescence, which affects tissue function, organismal health and lifespan. Studies in human and animal systems have shown that the length of telomere repeat regions correlate with phenotypic quality, and can even predict longevity, and reproductive success[2–4]. Telomere lengths are therefore an established biomarker for health and aging. Accelerated rates of telomere shortening have been correlated with multiple causes of stress such as diseases, harsh environments, faster growth rates and increased number of offspring[5–8]. Thus, telomere lengths have been used to assess the effects of different environmental and physiological stressors and to identify fitness trade-offs. The ability to monitor the well-being of organisms and to predict their future success has important applications in

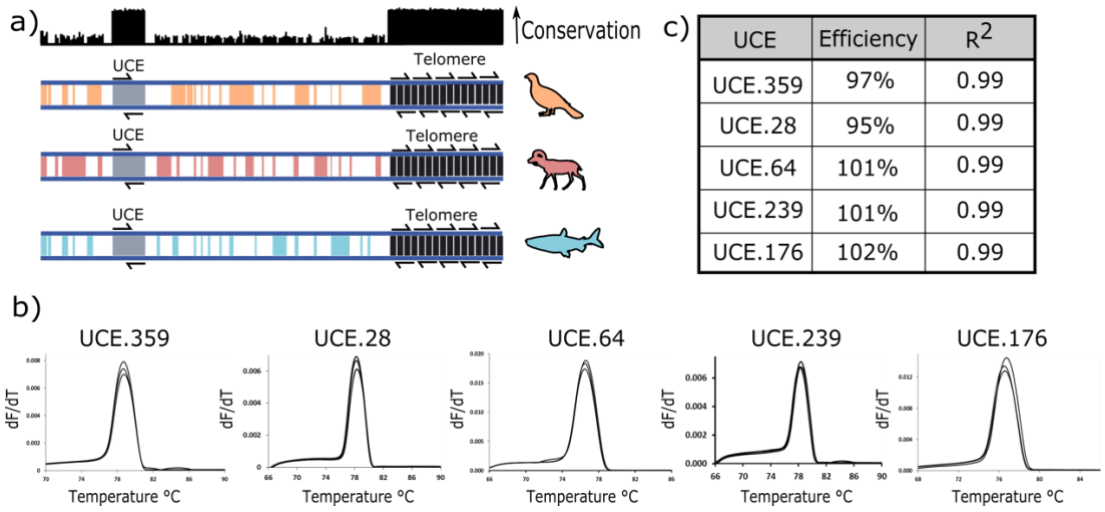
captive and wild systems, for both applied and basic research. Understanding telomere dynamics across the metazoan tree of life could also lead to a better understanding of the mechanisms and evolution of aging in general.

Despite this broad potential, the use of telomere length measurements has been limited to relatively few organisms, with the majority in bird species. One cause of this limited use is methodological challenges. Real-time quantitative PCR (qPCR) is one of the most widely used methods to measure telomere lengths[9]. Measurement of telomere length by qPCR requires primers that amplify telomere repeats, where the concentration of telomeric DNA in a sample is proportional to telomere length. The assay requires reference primers that amplify a non-telomeric region of the genome of interest to normalize for the amount of DNA in the sample. However, reference primers optimized in one species may not work in other species requiring the design and optimization of new primers for each new species that is to be studied. We note that repeating DNA sequence of telomeres is identical in all chordates, so the telomere specific primer pairs do not need to be species specific. However, reference primer development is especially challenging in non-model organisms with genomes that have not been sequenced and development of these primers is a key bottleneck in the use of telomere length measurements to monitor organisms of ecological and economic interest.

We here report reference primers optimized for qPCR in all chordates to broaden the use of telomere length measurements. We designed primer pairs that amplify regions of ultra-conserved elements (UCEs) – regions of DNA that were original found to be 100% identical in human, rat and mouse (Fig. 1a)[10]. We reasoned that reference primer pairs to these UCEs might be viable in all vertebrates and basal chordates, eliminating the need to design species-specific reference primers for each new telomere assays. Our goal was to find one primer pair for several UCEs that showed high amplification efficiency and a single-peak melt curve which indicates and single PCR product produced. We chose five UCEs from UCbase 2.0 and designed multiple primer pairs for each using a primer design

tool (OligoArchitect Sigma-Aldrich). During design, we limited the product length to 250 bps and designed primers to have a Tm of 60˚C to match the Tm of the already developed telomere primers[11]. The top four primer pairs for each of five UCEs, based on software ranking (Beacon Designer™), were synthesized for further testing. We initially tested the primer pairs on mouse DNA. We used melt-curve analysis to evaluate the specificity of each primer pair and chose a primer pair that gave a single melt peak (Fig. 1c). Amplification efficiencies of each primer pair were determined by performing a serial dilution of target DNA. We found an acceptable primer pair for all five UCEs that showed efficiencies within the best-practice range of 90-110% and correlation (R^2) of the Cq values for replicates of the standard curve that were greater than or equal to 0.980[12] (Fig. 1b). We chose one primer pair for each UCE for further investigation (Supplementary Table 1).

We next validated our primers across the vertebrate tree of life (Fig. 2a). We collected tissue from 19 species, including one basal chordate (sea squirt) for DNA extraction. We again used melt-curve analysis to evaluate the specificity of each primer pair in each organism. Surprisingly, we found that all five primer pairs amplified DNA from every organism with a single melt peak.



UCE	Efficiency	R^2
UCE.359	97%	0.99
UCE.28	95%	0.99
UCE.64	101%	0.99
UCE.239	101%	0.99
UCE.176	102%	0.99

Fig. 1 Primer design and quality control. a, Primers were designed to ultraconserved elements and telomere sequences which demonstrate conservation across chordate genomes. **b**, Efficiencies and R^2 values of each optimal UCE primer set. **c**, Melt curves of the best UCE primers from qPCR assays in mice.

An additional requirement for reference genes used for normalizing telomere length is that they must not vary in copy number between individuals in the population. For example, a duplication of a reference gene in some individuals would appear as a halving of their telomere lengths relative to a non-duplicated individual. The amplification of closely related homolog variants could also introduce normalization differences that would have the same effect as copy number variation. The challenge of choosing the best reference primers for telomere length measurements is similar to choosing primer pairs for normalizing real-time PCR data for gene expression studies where the goal is to find primers with the least variation between individuals in a population. We therefore evaluated our primer pairs using an algorithm (geNorm) designed for this purpose[13]. The geNorm algorithm evaluates the cumulative variation existing between multiple primer pairs and selects a combination of primer pairs that minimizes the overall variation within the sample array. Two or more primer pairs are preferred over a single primer pair to reduce random experimental variation. The geNorm algorithm iteratively eliminates UCE primer pairs that contribute the most variation until two final primer pairs are chosen. To test this approach, we extracted DNA from 17 to 20 individuals of mouse, ptarmigan, and northern goshawk, representing both sexes and a range of ages and tested with all five primer pairs.

Using the geNorm package in R, we were able to identify the best combination of UCE primer pairs for each of the tested organisms. We found that the use of two primer pairs for normalization leads to very low stability values in each of the organisms (Fig. 2b, Supplementary Fig. 1). Next, we extracted DNA from another mammal (big horn sheep) and tested the top three primer pairs found in mouse. Using geNorm we found low stability values (Fig. 2b). We measured a slightly lower correlation between the best two primers for sheep than other organisms, which is likely due to the more narrow distribution of low DNA concentrations extracted from these samples (Fig. 2c).We anticipate that these three primers would be a good starting place for any ungulate system. For investigators that wish to study telomeres in a

new organism, we recommend first testing all five UCE primer pairs on several (>10) individuals and performing a stability analysis. The low stability values observed support using the average DNA concentration for the best two primers to normalize telomere ratios.

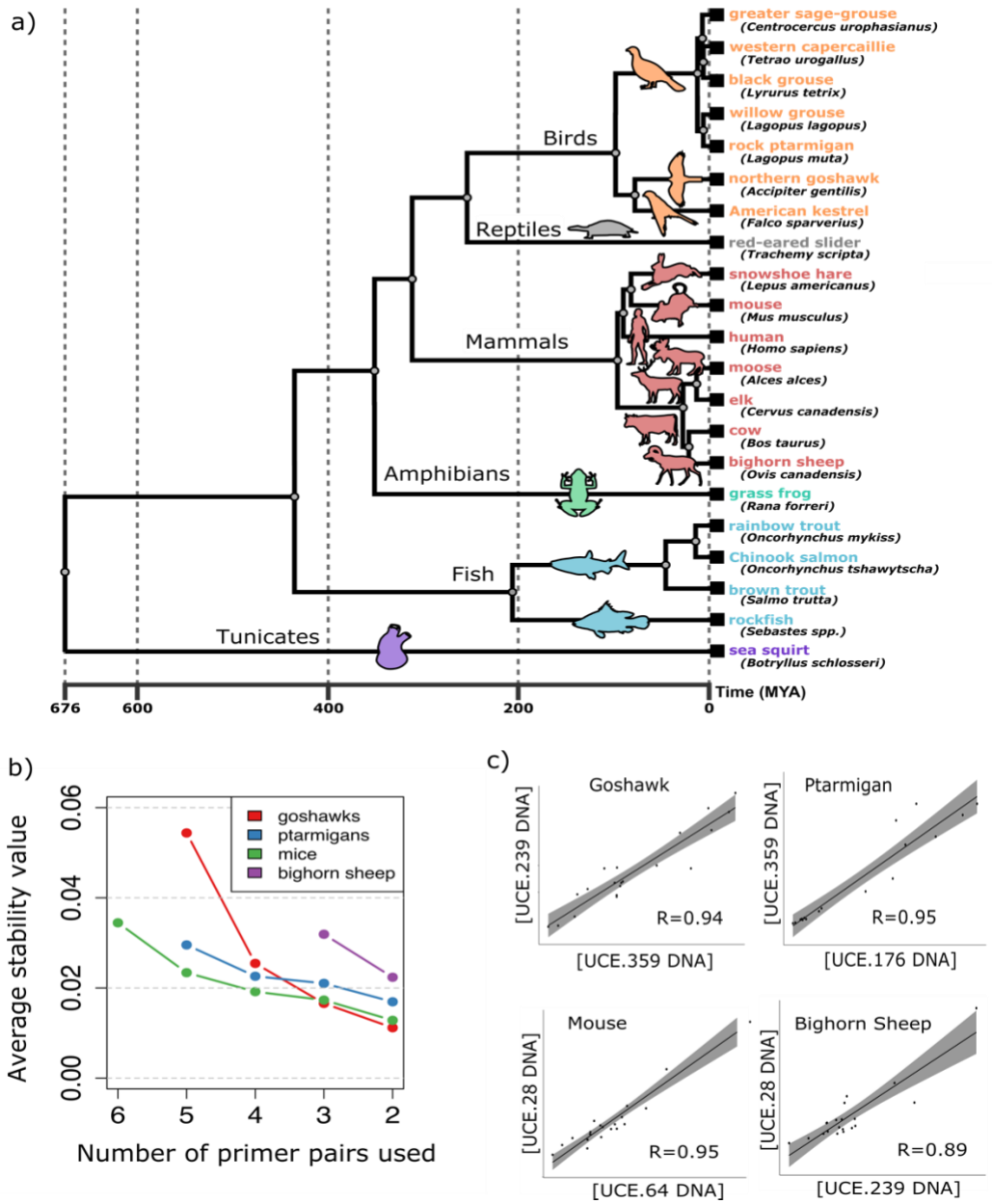


Fig. 2 Determining primer pairs across chordates. a, Phylogenetic tree of organisms in which reference primers have been validated. **b**, Average stability values generated in geNorm from the utilization of six to two reference gene primers. **c**, Pearson correlation from the DNA concentrations found using the two best primer pairs for four organisms as determined by geNorm stability values.

We also compared our best two UCE primer pairs for mouse to primer pairs previously reported in the literature for qPCR-based telomere assays in mice[14]. The previously reported primers target the acidic ribosomal phosphoprotein PO (36B4) gene and have been used in multiple publications. We found that our UCE primer pairs are an improvement compared to previously used genes because they show a single melt-peak while the 36B4 primers have wide multimodal melt peaks, which indicates mispriming or other PCR artifacts (Supplementary Fig. 2). Also, we note that the 36B4 primer pair was the second pair eliminated by the geNorm software, indicating that our top primer pairs are an improvement with respect to stability values in mice. These results suggest that our UCE reference primers may be useful even in organisms with established reference primers.

Telomere length measurements are an important tool for monitoring the demographics and well-being of animal populations. Because telomere lengths have been shown to predict lifespan and reproductive success, telomere lengths may be considered a proxy for fitness. For organisms of conservation concern, the telomere lengths have been shown to be an early indicator of extinction risk[15]. The UCE primer pairs reported here should facilitate robust measurements of telomere lengths in vertebrate and chordate study systems, even when limited or no genomic sequence data is available. It will be important to establish appropriate tissue collection protocols for each new organism because telomere dynamics vary between tissue types[16]. In addition, the biological implications of shorter telomere lengths in each organism must be determined. Nevertheless, understanding telomere length dynamics in more organisms increases the number of tools that can be used to address questions about stressors and will lead to a better general understanding of the evolutionary, environmental and mechanistic aspects of aging across the tree of life.

Acknowledgements

S.F.H., E.P.H., J.D.B., S.J.B., D.P.B. and E.J.H. were funded by two NSF awards #1826801 and #1807809, as well as an SRC #2018-SB-2842 and NASA award #80NSSC17K0738. J.S.F. was funded by an NSF EPSCoR OIA award #1826801 and K.R.C. and J.A.H. were supported by an NSF REU award DBI: 1263167 and DEB: 1145552. We would also like to thank Boise State University's Raptor Research Center, the Department of Biological Sciences, College of Arts and Sciences, and Division of Research, American kestrel nest box adopters, and landowners who have nest-boxes on their property as well as the Minidoka Ranger District of Sawtooth National Forest.

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